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(73) Proprietor: **MERCK & CO. INC.**
126, East Lincoln Avenue P.O. Box 2000
Rahway, New Jersey 07065 (US)

(72) Inventor: **Kang, Kenneth S.**
5590 Candlelight Drive
LaJolla, California 92037 (US)
Inventor: **Colegrove, George T.**
5238 Fontaine Street
San Diego, California 92120 (US)
Inventor: **Veeder, George T.**
4494 Pocahontas Avenue
San Diego, California 92117 (US)

(74) Representative: **Crampton, Keith John Allen et al,**
D YOUNG & CO 10 Staple Inn
London WC1V 7RD (GB)

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Heteropolysaccharides produced by bacteria and derived products, their preparation, and the lyophilized culture of the bacteria

This invention relates to polysaccharides, their production and compositions containing them.

It is known that heteropolysaccharides can be produced by certain microorganisms. Some of such heterpolysaccharides function as hydrophilic colloids and because of their viscosity and rheological properties have been used as thickening agents for aqueous systems.

As with other fields of technology, research has continued with the objective of discovering new heteropolysaccharides having useful properties as thickening, suspending and/or stabilizing agents.

The novel heteropolysaccharide of the present invention is produced by the action of a specified bacterium on a selected carbon source and fermentation medium ingredients under controlled conditions. The heteropolysaccharide of this invention is a high-molecular-weight polysaccharide containing primarily carbohydrate residues and a minor amount of protein. It is sometimes referred to as a "gum" but it is believed that the heteropolysaccharide terminology is more accurate and precise. In the following description of our invention, it will sometimes be referred to as Heteropolysaccharide 60, or S-60.

In accordance with the invention, the novel compound is prepared by fermentation of a suitable nutrient medium with a hitherto undescribed organism, based on extensive taxonomic studies, which is an unnamed *Pseudomonas* species. An unrestricted permanent deposit of this organism was made with the American Type Culture Collection on November 21, 1978 under Accession No. ATCC 31461. A lyophilized culture of this forms another aspect of the invention.

Various classification keys for the genus *Pseudomonas* and the culture description of *Pseudomonas* species are found in the 7th Edition of Bergey's Manual (Breed et al., (1957) and the 8th Edition of Bergey's Manual (Doudoroff et al., (1974), as well as by other schools in various publications; Hugh and Gilardi, 1974, Pseudomonas, *Manual of Clinical Microbiology*, 2nd ed., Lennette et al., Eds., pp. 250—269. American Society for Microbiology, Washington, D.C.; Weaver et al., 1972, Identification of Unusual Pathogenic Gram-Negative Bacteria, E. O. King, Center for Disease Control, Atlanta; Iizuka et al., 1963, Attempt at Grouping the Genus Pseudomonas, *J. Gen. Appl. Microbiology* 9:73—82; and Hendric et al., 1966, Identification of Certain Pseudomonas Species, *Identification Methods for Microbiologists*, Part A, Gibbs et al., Eds., pp. 1—7, Academic Press, New York.

These keys and descriptions were searched for a *Pseudomonas* species having morphological and cultural characteristics similar to those of ATCC 31461. The following considerations make the assignment of a new *Pseudomonas* species justified and necessary.

Description of the strain

1. Characteristics of cell morphology

Single cells, straight or often curved, generally 0.6—0.8 by 2.0—3.0 μm, often with tapered end. The older cultures become larger and longer (0.8—1.0 by >3 μm), misshaped cells and pleomorphism appear, especially on media with limited amount of carbohydrates. On the contrary, cells keep rather consistent rod shapes when grown on media with carbohydrates, but again, most cells become large and pleomorphism develops during prolonged incubation. Gram-negative, non-capsulated, poly-β-hydroxybutyrate and polyphosphate granules are seen especially in cultures of nitrogen-deficient media. Motile by polar multitrichous flagellation; one to four flagella are inserted at the polar end and occasionally subpolar insertion may be seen.

2. Characteristics of colonial morphology

On nutrient agar plates, small (0.8—1.1 mm in diameter) and large (3.2—3.5 mm in diameter) colonies appear. They are yellow carotenoid pigmented, smooth, round, and convex to pulvinate. Large colonies often have a concentric wrinkle. The surface of these colonies has a hard but not viscid texture and entire colonies are removed if pushed by a loop. On YM agar plates, only one type of relatively large (~6—7 mm in diameter) yellow, round, smooth, slimy and convex colonies appear. Slimy elastic membranes form on the surface of these colonies and whole surface membranes (of colonies) can be removed. The secondary growth may occur around the edge of the original colonies. The color of these colonies is darker yellow towards the center than the edge and concentric color formation appeared. In addition to the intra-cellular yellow carotenoid pigment(s), diffusible brown pigment developed as a result of auto-oxidation after prolonged incubation. This phenomenon is more easily recognized on Nutrient agar. No fluorescent pigment was produced.

3. Physicological and biochemical characteristics

The growth range of the strain S-60 is about 20°C to 41°C. No growth occurs at 4°C. 3.0% NaCL is sufficient to inhibit the growth and the strain is capable of growth at pHs between 5 and 11.

Acid, but no gas, is produced from almost all carbohydrates but not from polyalcohols. Urease may be produced. MR, VP, and indole tests were all negative. Argine dihydrolase, lysine and ornithine decarboxylase are not produced. Acid and reduction occurs in litmus milk. Lipolytic egg yolk reaction is negative. Gelatin is weakly hydrolyzed but not casein, starch, alginate, pectin, cellulose, chitin and DNA.

4. Susceptibility to antibiotics

The strain is very susceptible to Kanamycin, Neomycin, Chlortetracycline, Erythromycin, but not Streptomycin and Penicillin.

5. Nutritional characteristics

Organic growth factors are not required and ammonium salts serve as sole nitrogen source. At least 35 organic compounds were utilized, those being most carbohydrates other than D-ribose, starch, 2-ketogluconate, and mucate. In addition, acetate, caproate, caprylate, pelargonate, succinate, azelate, L-malate, DL-β-hydroxybutyrate, pyruvate, ethanol, n-propanal, p-hydroxybenzoate, phenylacetate, L-α-alanine, L-threonine, L-leucine, DL-isoleucine, L-aspartate, L-glutamate and L-tyrosine were utilized.

6. The G+C content of the DNA

Evaluation of the DNA resulted in the mole % to be ~68 (by Tm).

TABLE 1

Biochemical and other miscellaneous tests employed for the strain S-60

Oxidase: Kovac's	+(weak)	Hydrolysis of:	
Pathotech	+(weak)	Gelatin	+(weak)
		Casein	–
Catalase	+	Starch	–
OF medium: Oxidative	+	Tween 80	+
Fermentative	–	Pectin	–
Gas from glucose	–	Alginate	–
H_2S production: TSI	–	Cellulose	–
from cystine	+	Chitin	–
Ammonium from peptone	+	DNA	–
β-Galactosidase (ONPG)	+(API)	Esculin	+
Arginine dihydrolase	–		
Lysine decarboxylase	–	Growth on various media:	
Ornithine decarboxylase	–	EMB agar	–
Tryptophan deaminase	–	MacConkey agar	–
Phenylalanine deaminase	–	SS agar	–
Urease	+/±	Mannitol salt agar	–
Indole	–	TCBS agar	–
MR test	–	Tinsdale tellurite	
VP test	–	blood agar	+
Nitrate reduction	–	Pseudosel agar	–
Nitrite reduction	–		
Denitrification	–	Pigment production:	
N_2-fixation		King A medium	–
Growth in Burk's medium	+	King B medium	–
Nitrogenase activity	–		
Malonate (Oxidation)	–	Dye reaction:	
Phosphatase	+	Congo red	–
Haemolysis (sheep blood)	–	Nite blue	–
Litmus milk: acid,			
reduction only		TSI: Slant	color no change
3-ketolactose production	–	Butt	color no change
Survival at 60°C for		Gas	–
30 min.	–	Egg Yolk Reaction	–

Fermentation conditions

Heteropolysaccharide S-60 is produced during the aerobic fermentation of suitable aqueous nutrient media under controlled conditions via the inoculation with the organism of the unnamed *Pseudomonas* species. The media are usual media, containing sources of carbon and nitrogen and inorganic salts.

In general, carbohydrates (for example, glucose, fructose, maltose, sucrose, xylose or mannitol can be used either alone or in combination as sources of assimilable carbon in the nutrient medium. The exact quantity of the carbohydrate source or sources utilized in the medium depend in part upon the other ingredients of the medium but, in general, the amount of carbohydrate usually varies from 2% to 4% by weight of the medium. These carbon sources can be used individually, or several such carbon sources may be combined in the medium. In general, many proteinaceous materials may be used as nitrogen sources in the fermentation process. Suitable nitrogen sources include, for example, yeast

hydrolysates, primary yeast, soybean meal, cottonseed flour, hydrolysates of casein, corn steep liquor, distiller's solubles and tomato paste. The sources of nitrogen, either alone or in combination, are used in amounts ranging from about 0.05% to 0.2% by weight of the aqueous medium.

Among the nutrient inorganic salts which can be incorporated in the culture media are the customary salts capable of yielding sodium, potassium, ammonium, calcium, phosphate, sulfate, chloride, carbonate, and like ions. Also included are trace metals such as cobalt, manganese, iron and magnesium.

It should be noted that the media described in the examples are merely illustrative of the wide variety of media which may be employed, and are not intended to be limitative.

The fermentation is carried out at temperatures ranging from about 25°C to 35°C; however, for optimum results it is preferable to conduct the fermentation at temperatures of from about 28°C to 32°C for from 40 to 60 hrs. The pH of the nutrient media for growing the *Pseudomonas* culture and producing the polysaccharide S-60 can vary from about 6 to 8.

Although the novel polysaccharide S-60 is produced by both surface and submerged culture, it is preferred to carry out the fermentation in the submerged state.

A small scale fermentation is conveniently carried out by inoculating a suitable nutrient medium with the culture and, after transfer to a production medium, permitting the fermentation to proceed at a constant temperature of about 30°C on a shaker for several days.

The fermentation is initiated in a sterilized flask of medium via one or more stages of seed development. The nutrient medium for the seed stage may be any suitable combination of carbon and nitrogen sources. The seed flask is shaken in a constant temperature chamber at about 30°C for 1—2 days, or until growth is satisfactory, and some of the resulting growth is used to inoculate either a second stage seed or the production medium. Intermediate stage seed flasks, when used, are developed in essentially the same manner; that is, part of the contents of the flask from the last seed stage are used to inoculate the production medium. The inoculated flasks are shaken at a constant temperature for several days, and at the end of the incubation period the contents of the flasks are recovered by precipitation with a suitable alcohol such as isopropanol.

For large scale work, it is preferable to conduct the fermentation in suitable tanks provided with an agitator and a means of aerating the fermentation medium. According to this method, the nutrient medium is made up in the tank and sterilized by heating at temperatures of up to about 121°C. Upon cooling, the sterilized medium is inoculated with a previously grown seed of the producing culture, and the fermentation is permitted to proceed for a period of time as, for example, from 2 to 4 days while agitating and/or aerating the nutrient medium and maintaining the temperature at about 30°C. This method of producing the S-60 is particularly suited for the preparation of large quantities.

The product is recovered from the fermentation medium by precipitation with a suitable lower alcohol, such as isopropanol.

Physical and chemical properties of the polysaccharide S-60

The heteropolysaccharide produced by an unnamed *Pseudomonas* species is composed of about principally carbohydrate, 3—4.5% acetyl groups as the *O*-glycosidicallyl linked ester, and 10—15% protein.

The carbohydrate portion of the S-60 polysaccharide contains uronic acid (about 12% b.o. wt. gum) and the neutral sugars glucose and rhamnose. The approximate molar ratio of rhamnose to glucose is 1.5 to 1.

The acetyl content of 4.5% was determined by treating a 0.2% aqueous solution of S-60 gum with an alkaline, hydroxylamine reagent followed by treatment with an acidic ferric chloride reagent [S. Hestrin (1949) *J. Biol. Chem. 180* 249—261].

The neutral sugars of polysaccharide S-60 were determined by dissolving ten mg of the product in 2 ml of 2 N H_2SO_4, and the mixture is heated at 100°C for 4 hours. The resulting solution is cooled, neutralized with barium hydroxide and the pH is brought to 5—6 with solid carbon dioxide. The resulting precipitate of barium sulfate is removed by centrifugation and the supernatent is concentrated to a syrup under reduced pressure. The sugars in the hydrolysate are tentatively identified by gas-liquid chromatography of their aldononitril acetate derivatives on a Hewlett-Packard Model 5750 chromatograph using 3% by weight OV-225 on 80/100 U.S. Standard mesh Gas Chrome Q at 210°C. The sugars are identified and quantitated by comparison with authentic standards [J. K. Baird, M. J. Holroyde, and D. C. Ellwood (1973) carbohydr. Res. *27* 464—467].

The various neutral sugars of the polysaccharides were also characterized by use of descending paper chromatography on Whatman No. 1 chromatography paper using as the solvent the upper layer of pyridine:ethyl acetate:water (2:5:5 by volume). Chromatograms were stained using silver nitrate dip and acid aniline phthalate spray reagent. Component sugars were identified by co-chromatography with sugar standards and by the specific-color reaction with the aniline phthalate reagent.

The uronic acid content of the polysaccharide was determined by two separate methods. In one method the sample was decarboxylated with 19% hydrochloric acid and the liberated carbon dioxide was trapped in standard sodium hydroxide and determined by back titration [B. L. Browning (1967)

Methods of Wood Chemistry *II*, 632—633] and by the carbazole colorimetric method [T. Bitter and H. M. Muir (1962) Anal. Biochem. *4* 330—334].

Paper electrophoresis was used for the separation and tentative identification of the uronic acids present in the neutralized acid hydrolysate described above. Aliquots of this and known uronic acid standards were applied to Camag electrophoresis paper No. 68-011 and electrophoresis was carried out for 2.0 hours in a pH 2.7 buffer using a Camag Model HVE electrophoresis apparatus. Chromatograms were air dried and stained with silver nitrate dip reagent to locate the uronic acids being separated. Two major and one minor uronic acid spots were found. One of the major spots migrated with the same mobility as glucuronic acid (R_{GlcA}=1.0) while the other major spot (R_{GlcA}=0.85) and the minor spot (R_{glcA}=0.73) had lower mobility. Under these same conditions the relative mobility of known uronic acids are:

	R_m
Glucuronic acid	1.0
Mannuronic acid	0.96
Galacturonic acid	0.65
Guluronic acid	0.63

An infrared spectrum of native S-60 was made on dried material in a KBr pellet. The heteropolysaccharide evidenced peaks at: 3400 cm^{-1}, 2950 cm^{-1}, 1740 cm^{-1} and 1620 cm^{-1} indicated hydroxyl groups, methylene groups, carbonyl groups and carboxylic acid salts. It is incompatible with methylene chloride dye, and is substantially insoluble in N,N-dimethylformamide, soluble in DMSO or formamide.

A sample of S-60 shows the following elemental analysis: N—2.00%, C—42.62%, H—5.80%.

The polysaccharide S-60 imparts viscosity to an aqueous medium when dissolved in water in low concentrations. Because of this, its sensitivity to shear and overall rheology, it is useful as a thickening, suspending and stabilizing agent in aqueous systems; for example, as an additive to textile printing pastes or in formulating low drift aqueous herbicide compositions, salad dressings, thickened puddings, and adhesive compositions. After heating and cooling, it forms a weak very elastic gel.

Deacetylated, non-clarified S-60

When the dry polymer or the fermentation broth is heated at high pH (using e.g., sodium carbonate or sodium hydroxide to pH 10) and high temperatures 90—100°C for 10 minutes to 45 minutes deacetylation readily occurs. The resultant deacetylated polysaccharide S-60 forms firm non-elastic or brittle gels, useful in many industrial and food applications. The composition of the deacetylated S-60 is principally carbohydrate, about 17% protein, and 0% acetyl. The carbohydrate portion comprises about 13% uronic acid and the neutral sugars rhamnose and glucose in the approximate molar ratio 1.5:1.

One use for the deacetylated gum results from the fact that as a rigid, brittle gel, it can be molded, and used as a rigid structure, which after treatment with a suitable solution such as fragrance, finds applicability as a room deodorant or an air freshener, or the like. The deacetylated gum can also be used in gel electrophoresis, as a gelling agent for use in microtomes in electronmicroscopy. The native and deacetylated gums can also be used as suspending agents for barium in radiology, confectionery products, and as impression materials in tool making, dentistry and criminology.

An infrared spectrum of deacetylated S-60 made on dried material in a KBr pellet evidenced peaks at: 3400 cm^{-1}, 2950 cm^{-1}, 1740 cm^{-1}, 1650 cm^{-1}, and 1610 cm^{-1}.

A sample of deacetylated S-60 shows the following elemental analysis: N—2.67%, C—41.89%, H—6.07%.

Deacetylated, clarified S-60

The composition of the clarified, deacetylated S-60 is as follows: about 2% protein, 0% acetyl, and carbohydrate, the latter being about 22% uronic acid and the neutral sugars rhamnose and glucose in the approximate molar ratio 1.5 to 1.

In infrared spectrum of clarified, deacetylated S-60 made on dried material in a KBr pellet evidenced peaks at: 3400 cm^{-1}, 2950 cm^{-1}, and 1600 cm^{-1}.

A sample of deacetylated, clarified S-60 shows the following elemental analysis: N—0.42%, C—36.85%, H—5.62%. The sample has a specific rotation:

$$[\alpha]^{25}_{589} = -45°$$

The deacetylated clarified gum is especially useful as an agar substitute in microbiological culture media for various clinical and non-clinical microorganisms using a wide variety of culture media. The concentration of deacetylated clarified gum necessary to replace agar is dependent upon the medium

used, but is within the range of about 0.5 to about 1.25% (weight volume). Growth characteristics of microorganisms are quite similar to those on standard agar-based media.

The following detailed examples illustrate representative aspects of this invention.

Example 1

Fermentation procedure for producing heteropolysaccharide S-60

A. Culture maintenance

The unnamed *Pseudomonas* organism, ATCC 31461, grows quite well on NA or YM agar, which are used routinely for culture maintenance. The incubation temperature is 30°C. The organism produces a yellow-orange carotenoid pigment and a brown diffusable pigment by 2—5 days incubation.

B. Seed preparation

Flask seeds are prepared in YM broth incubated at 30°C. When inoculated with a fresh plate culture, the YM broth cultures give good growth and gum formation by 24 hours.

The fermentation seed medium is the same as final fermentor medium, using one-gallon fermentors as seed vessels.

C. Final fermentor medium

The sodium- and potassium-salt form of the gum are prepared in a different media; both of which are described below. The organism has a definite K+ requirement which must be added to the sodium fermentation medium. (3% dextrose can also be used). Also, 0.2% corn steep liquor is added in both media.

Sodium salt	Potassium salt
3.0% Glucose	3.0% Glucose
0.01% $MgSO_4 . 7H_2O$	0.01% $MgSO_4 . 7H_2O$
0.09% NH_4NO_3	0.09% NH_4NO_3
0.05% Promosoy (soy protein concentrate)	0.05% Promosoy
1 ml/L HoLe salts	1 ml/L HoLe salts
1 ppm Fe++	1 ppm Fe++
0.05% Na_2HPO_4	0.05% K_2HPO_4
10 ppm K+	
pH control=NaOH	pH control=KOH

HoLe salts are a trace element solution containing, tartrate, magnesium molybdate, $CoCl_3$, $ZnCl_2$, $CuCl_2$, boric acid, manganese chloride and ferrous sulfate.

When a low calcium product is desired, either of the media above are used with deionized water. Fermentation is complete aq. 50 hours; beer viscosity usually is 5000—8000 mPas.

D. Recovery

Because of the gelling nature of the product, good fiber formation usually does not occur with ambient precipitation. However, we have found that by pasteurization at 90—95°C for 10—15 minutes (during which the thick beer heat-thins considerably) excellent fibers can be obtained by precipitation of the beer using two volumes of 99% isopropanol per volume of beer without cooling. Average yields of 1.5% gum are obtained with 3% glucose in the 20L and 70L fermentors.

E. Drying

The product is recovered and dried at 50—55°C for up to one hour in a forced-air tray dryer.

F. Product quality

One-percent viscosities of the K+ salt are usually in the range of 3000 cps and for the low-calcium sodium salt, approximately 7000 cps.

Example 2

Deacetylation and clarification of the heteropolysaccharide S-60

Clarification of the gum, while not necessarily for all uses, is of value when the gum is used as an agar substitute. Clarification can be accomplished before deacetylation (in the native state) or after deacetylation. Since deacetylation uses hot caustic, and clarification is done hot, the two procedures are easily and conveniently combined. Both deacetylation and clarification can be done with the beer or the dry polymer. For deacetylation, if the beer is used, the pH is adjusted to 10 with KOH, the solution

heated to 90°C for 15 minutes, the pH adjusted to 7 with dilute H_2SO_4, then $CaCl_2$ is added to a 0.2% concentration and cooled. A hard brittle gel results.

The general procedure for both deacetylation and clarification follows:

A. A 2% solution of beer or gum is heated to 90°C.

B. The pH is adjusted to 10 with KOH.

C. The temperature of the beer or solution was maintained at 90—95°C for 15 minutes.

D. The pH is adjusted to 6—8 with dilute HCl or H_2SO_4.

E. Ten gms/liter of Super Aid were added to the material to be filtered.

F. The material was filtered through a pressure filter unit (pre-heated) with approximately a 6 mm bed of Super Aid and approximately 20—30 psi (1.38—2.06 bar), using a filter unit with an area of 136 cm^2.

G. The filtrate is precipitated with isopropanol immediately to prevent gelation and the fibers dried at 50°C for one hour or less.

When no deacetylation is necessary, the above procedure is followed, except that the pH is not raised; rather than holding at 90°C, the solution is immediately filtered, and then recovered.

Clarification is always done on the potassium form; KCl can be added to a solution of previously made product as necessary.

Example 3

Heteropolysaccharide S-60 gelling characteristics

A compilation of data comparing the native gum and the deacetylated gum, both in the K+ form and in the Ca++ form, with carrageenan and agar follows:

Type	Gel nature	Melts	Sets	Hysteresis
Native S-60	Very Elastic	65—70°C	65—70°C	None
Deacetylated S-60				
K^+ Gel	Brittle	90°C	31—46°C	45—60°C
Ca^{2+} Gel	Brittle	90°C	45—50°C	45—50°C
Kappa Carrageenan	Brittle	40—95°C	25—75°C	15—20°C
Agar*	Brittle	60—97°C	32—39°C	60°C

*Bacteriological grade specs: gelling temperature range 33°—39°C, melting temperature 70°C minimum. (Whistler's "Industrial Gums").

Minimum gelling concentration

Kappa Carrageenan — 0.3%.

Agar — 0.04%.

Deacetylated S-60 — 0.05%.
(calcium gel).

Note above that there is a wide range of temperatures given for setting and melting of all the various types of gels. For agar, the variations are primarily due to type of seaweed while for kappa carrageenan the potassium ion concentration determines the gel characteristics. The gels of deacetylated S-60 are primarily characterized by the degree of deacetylation. With only slight deacetylation the gels set at higher temperatures and are more elastic; in fact, a wide range of gel types from very elastic to very brittle is possible, depending on the degree of deacetylation. The gels appear to be more similar to agar than to kappa carageenan, primarily because of the large hysteresis between setting and melting temperatures. It should be emphasized that they are difficult to melt and the gel-sol transition is difficult to observe. On the other hand, the gelling temperatures can be easily defined since the gels set sharply within a few degrees from incipient gelation to solid gel.

Example 4

Deacetylated, non-clarified S-60

S-60 fermentation liquor is heated to 90°C and the pH adjusted to 10 by addition of 25% KOH. The temperature is maintained for 15 minutes, followed by neutralization with concentrated HCl. This liquor is then drained from the fermentor while hot and recovered with 2 volumes of 99% IPA. The fibers of deacetylated S60 are collected, dried at 55°C for one hour in a forced air tray drier, and then milled to a powder.

Example 5

Clarification of deacetylated S-60

Deacetylated heteropolysaccharide S-60 as prepared in Example 4 is reconstituted to a 1% concentration in deionized water using a Lightnin mixer for one hour followed by mixing on an Arti-Barinko with heating to 50°C. The solution is then centrifuged at 10,000 R.P.M. (GSA head) for 20

minutes in a Sorvall RC2-B refrigerated centrifuge keeping the temperature above 40°C. The supernatant is decanted off and sequentially filtered through Gelman type AN hydrophilic Acropor membranes (293 mm) of porosities of 5 μm, 3 μm, 1.2 μm and 0.8 μm. The filtrate is added to approximately 3—4 volumes of 99% isopropanol, the fibers collected and dried briefly in the forced air tray drier at 55°C, followed by milling to a powder. The product is the deacetylated, clarified S-60 gum of this invention.

Example 6

Agar replacement using deacetylated clarified S-60

Several different media are prepared as follows:

Nutrient agar

(A) 0.8% Nutrient Broth (Difco)
1.5% Agar (Difco)

(B) 0.8% Nutrient Broth (Difco)
0.2% KCl
0.9% S-60

Trypticose soy agar

(A) 2.75% Trypticose Soy Broth (BBL)
1.5 % Agar (Difco)

(B) 2.75% Trypticose Soy Broth (BBl)
0.2% KCl
0.9% S-60

Potato dextrose agar

(A) 2.4% Potato Dextrose Broth (Difco)
1.5% Agar

(B) 2.4% Potato Dextrose Broth (Difco)
0.2% KCl
0.9% S-60

YM agar

(A) 2.1% YM Broth (Difco)
1.5% Agar (Difco)

(B) 2.1% YM Broth
0.2% KCl
0.9% S-60

Brain heart infusion agar

(A) 3.7% BHI Broth (Difco)
1.5% Agar

(B) 3.7% BHI Broth (Difco)
0.2% KCl
0.9% S-60

Burk's agar

(A)	(B)
0.0584% K_2HPO_4	—Same as (A)
0.0225% KH_2PO_4	
0.0174% K_2SO_4	
0.0164% $MgCl_2 . 6H_2O$	
0.0064% $CaCl_2 . 2H_2O$	
0.0005% $FeCl_3 . 6H_2O$	
0.00002% $Na_2MoO_4 . 2H_2O$	
0.0116% NaCl	
1.0% Glucose	
1.5% Agar (Difco)	0.2% KCl 0.9% S-60

Deionized water was used for all media. The ingredients were combined (except for Burk's) and autoclaved for 15—20 minutes at 121°C and 15 psi (1.03bar), cooled to 55°C, and poured into sterile petri dishes. The ingredients for Burk's were combined, except for the glucose, which was autoclaved separately, and added to the medium after autoclaving. After these nutrient plates had solidified and

allowed to incubate at ambient temperature for 24 hours to check for sterility, they were streaked with the following fourteen cultures:

Agromyces ramosus ATCC 25173
Arthrobacter globiformis ATCC 8010
Aureobasidium pullulans NRRL YB-3861
Azotobacter indicus var *myxogenes* strain S-7 ATCC 21423
Azotobacter vinelandii ATCC 9047
Beijerinckia lacticogenes ATCC 19361
Erwinia cartovora ATCC 8061
Escherichia coli strain EG-47
Klebsiella pneumoniae strain S-53
Nocardia salmonicolor ATCC 21243 S-60
Streptococcus faecalis
Trichoderma longbrachiatum ATCC 13631
Zoogloea ramigera ATCC 25935

The plates were incubated at 30°C for 3—5 days and then examined for growth. Good growth was observed for all strains on media made with S-60 and little difference in colonial morphology was noted between media made with S-60 instead of agar. These results indicate that S-60 is an excellent replacement for agar in microbiological media. The gel point for all media containing S-60, except BHI medium and TSA, was 42°C. The gel point for BHI agar and TSA was 52°C. Agar typically gels at 42—44°C.

Example 7

Preparation of molded scented gels using deacetylated S-60

(A) 1.50% Polysaccharide S-60
0.75% Sodium Carbonate
0.025% Methyl p-hydroxybenzoate
3.00% Rose fragrance
4.00% Isopropanol
2.00% Ethylene glycol
88.50% Water

The native polysaccharide S-60 is blended with sodium carbonate and preservative and dissolved in water at 70°C. The solution is then further heated to 90°C and held at that temperature for ten minutes to deacetylate the polysaccharide. After cooling to 60°C, the fragrance dispersed in the solvents is added and the mixture placed in the usual air freshener plastic molds. When the mixture cools to 38°C, gelation occurred and a firm, self-supporting gel with good fragrance releasing properties results.

(B) A dry deacetylated polysaccharide S-60 is prepared from the fermentate beer by adjusting the pH to 10.0 with dilute sodium hydroxide and heating to 90°C for fifteen minutes. The solution is neutralized to pH 7 with dilute hydrochloric acid, precipitated in two volumes of isopropanol, dried, and milled. A solid air freshener gel is prepared from the deacetylated product in the following manner:

3.0 grams of the deacetylated polysaccharide S-60 are blended with 1.5 grams potassium chloride and 0.15 grams methyl p-hydroxybenzoate preservative and added to 177 ml water. The solution is heated to 90°C to dissolve; cooled to 60°C, and a blend of 6.0 grams peppermint oil fragrance, 8.0 grams isopropanol, and 4.0 grams ethylene glycol is added. The solution is placed in plastic molds and cooled to ambient temperature. A strong, brittle gel with heavy mint fragrance forms. The gel can be unmolded easily and retains its shape without sagging.

Claims

1. Heteropolysaccharide S-60, containing 10—15% protein, 3—4.5% acetyl and principally carbohydrate, the carbohydrate portion containing about 12% uronic acid, and rhamnose and glucose in the approximate molar ratio of 1.5:1, the said heteropolysaccharide being incompatible with methylene chloride dye, being substantially insoluble in N,N-dimethylformamide and soluble in DMSO or formamide, having an infrared spectrum characterized by peaks at 3400 cm^{-1}, 2950 cm^{-1}, 1740 cm^{-1} and 1620 cm^{-1}, and being obtainable by incubating the unnamed *Pseudomonas* organism ATCC 31461 in a fermentation medium containing a carbohydrate, a source of potassium ions, a source of nitrogen, trace inorganic elements and water at a temperature of 28—32°C and pH of 6—8 for 40—60 hours, and recovering the gum by precipitation with a suitable lower alcohol.

2. A process for producing heteropolysaccharide S-60 of Claim 1 comprising incubating the unnamed *Pseudomonas* organism ATCC 31461 in a fermentation medium containing a carbohydrate, a source of potassium ions, a source of nitrogen, trace inorganic elements and water at a temperature of 28—32°C and pH of 6—8 for 40—60 hours, and recovering the gum by precipitation with a suitable lower alcohol.

3. A process as claimed in Claim 2, in which the nitrogen source is corn steep liquor and the alcohol is isopropanol.

4. The lyophilized culture of an unnamed *Pseudomonas* species, ATCC 31461.

5. Deacetylated non-clarified heteropolysaccharide S-60, containing 17% protein, carbohydrate, and an acetyl content of 0%, the carbohydrate portion comprising 13% uronic acid and the neutral sugars rhamnose and glucose in the approximate molar ratio 1.5:1, and obtained by hydrolysis from the heteropolysaccharide S-60 claimed in Claim 1.

6. Deacetylated clarified heteropolysaccharide S-60, containing 2% protein, carbohydrate, and an acetyl content of 0%, the carbohydrate portion comprising 22% uronic acid and the neutral sugars rhamnose and glucose in the approximate molar ratio 1.5:1, and obtained from the heteropolysaccharide S-60 claimed in Claim 1.

7. A process for preparing the compound of Claim 5 which comprises heating a 1—5% aqueous solution of heteropolysaccharide S-60 as claimed in Claim 1 at a pH of about 10 and at a temperature of 90—100°C for from 10 minutes to 45 minutes, and recovering the product thereby produced.

8. A microbiological culture medium comprising 0.5—5% of the deacetylated product from the heteropolysaccharide S-60 claimed in Claim 1 and a nutrient.

9. A medium as claimed in Claim 8, in which the deacetylated heterpolysaccharide S-60 is clarified.

10. A scented gel composition comprising 0.1—5% of the deacetylated product from the heteropolysaccharide S-60 claimed in Claim 1, water and a lower alkanol.

Revendications

1. Hétéropolysaccharide S-60, contenant 10—15% de protéine, 3—4,5% d'acétyle et principalement un hydrate de carbone, la fraction hydrate de carbone contenant environ 12% d'acide uronique, et du rhamnose et du glucose dans un rapport molaire approché de 1,5:1, ledit hétéropolysaccharide étant incompatible avec un colorant au chlorure de méthylène, étant pratiquement insoluble dans le N,N-diméthylformamide et soluble dans le DMSO ou le formamide, ayant un spectre infrarouge caractérisé par des pics à 3400 cm^{-1}, 2950 cm^{-1}, 1740 cm^{-1} et 1620 cm^{-1}, et pouvant être obtenu en faisant incuber l'organisme *Pseudomonas* sans dénomonation ATCC 31461 dans un milieu de fermentation contenant un hydrate de carbone, une source d'ions potassium, une source d'azote, des oligo-éléments inorganiques et de l'eau à une température de 28—32°C et un pH de 6—8 pendant 40 à 60 h, et en recueillant la gomme par précipitation avec un alcool inférieur approprié.

2. Procédé de production de l'hétéropolysaccharide S-60 de la revendication 1, dans lequel on fait incuber l'organisme *Pseudomonas* sans dénomination ATCC 31461 dans un milieu de fermentation contenant un hydrate de carbone, une source d'ions potassium, une source d'azote, des oligo-éléments inorganiques et de l'eau à une température de 28—32°C et un pH de 6—8 pendant 40—60 h, et on recueille la gomme par précipitation avec un alcool inférieur approprié.

3. Procédé selon la revendication 2, dans lequel la source d'azote est la liqueur de maïs macérée et l'alcool est l'isopropanol.

4. La culture lyophilisée d'une espèce de *Pseudomonas* sans dénomination, ATCC 31461.

5. Hétéropolysaccharide S-60 non-clarifié désacétylé, contenant 17% de protéine, un hydrate de carbone, et une teneur en acétyle de 0%, la fraction hydrate de carbone comprenant 13% d'acide uronique et les sucres neutres rhamnose et glucose en un rapport molaire approximatif de 1,5:1, et obtenu par hydrolyse à partir de l'hétéropolysaccharide S-60 revendiqué dans la revendication 1.

6. Hétéropolysaccharide S-60 clarifié désacétylé, contenant 2% de protéine, un hydrate de carbone, et une teneur en acétyle de 0%, la fraction hydrate de carbone comprenant 22% d'acide uronique et les sucres neutres rhamnose et glucose dans le rapport molaire approximatif de 1,5:1, et obtenu à partir de l'hétéropolysaccharide S-60 revendiqué dans la revendication 1.

7. Procédé de préparation du composé de la revendication 5, dans lequel on chauffe une solution aqueuse à 1—5% de l'hétéropolysaccharide S-60 tel que revendiqué dans la revendication 1 à un pH d'environ 10 et à une température de 90—100°C pendant 10 à 45 minutes, et l'on recueille le produit ainsi préparé.

8. Milieu de culture microbiologique comprenant de 0,5 à 5% de produit désacétylé de l'hétéropolysaccharide S60 revendiqué dans la revendication 1 et un nitriment.

9. Milieu selon la revendication 8, où l'hétéropolysaccharide S-60 désacétylé est clarifié.

10. Composition de gel parfumée comprenant de 0,1 à 5% de produit désacétylé de l'hétéropolysaccharide S-60 selon la revendication 1, de l'eau et un alcanol inférieur.

Patentansprüche

1. Heteropolysaccharid S-60, enthaltend 10—15% Protein, 3—4,5% Acetyl und hauptsächlich Kohlenhydrat, wobei der Kohlenhydratanteil etwa 12% Uronsäure, und Rhamnose und Glukose im ungefähren Molverhältnis von 1,5:1 enthält, und das Heteropolysaccharid unverträglich mit Methylenchlorid-Farbstoff ist, im wesentlichen unlöslich in N,N-Dimethylformamid und löslich in DMSO oder Formamid ist, mit einem Infrarotsprektrum, charakterisiert durch Peaks bei 3400 cm^{-1}, 2950

cm^{-1}, 1740 cm^{-1} und 1620 cm^{-1}, und das erhältlich ist durch Inkubieren des namenlosen Pseudomonas-Organismus ATCC 31461 in einem Fermentationsmedium, das ein Kohlenydrat, eine Quelle für Kaliumionen, eine Quelle für Stickstoff, Spuren anorganischer Elemente und Wasser enthält, bei einer Temperatur von 28—32°C und einem pH von 6—8 während 40—60 Stunden, und Gewinnen des gummiartigen Produkts durch Ausfällen mit einem geeigneten niedrigen Alkohol.

2. Verfahren zur Herstellung des Heteropolysaccharids S-60 nach Anspruch 1 durch Inkubieren des namenlosen Pseudomonas-Organismus ATCC 31461 in einem Fermentationsmedium, das ein Kohlenhydrat, eine Quelle für Kaliumionen, eine Quelle für Stickstoff, Spuren anorganischer Elemente und Wasser enthält, bei einer Temperatur von 28—32°C und einem pH von 6—8 während 40—60 Stunden, und Gewinnung des gummiartigen Produkts durch Ausfällen mit einem geeigneten niedrigen Alkohol.

3. Verfahren nach Anspruch 2, bei dem die Stickstoffquelle Mais- bzw. Kornquellflüssigkeit ist und der Alkohol Isopropanol ist.

4. Lyophilisierte Kultur einer namenlosen Pseudomonas-Species ATCC 31461.

5. Deacetyliertes, nicht geklärtes Heteropolysaccharid S-60, enthaltend 17% Protein, Kohlenhydrat und einen Acetylgehalt von 0%, wobei der Kohlenhydratanteil 13% Uronsäure und die neutralen Zucker Rhamnose und Glukose im ungefähren Molverhältnis 1,5:1 enthält, und erhalten durch Hydrolyse des im Anspruch 1 beanspruchten Heterpolysaccharids S-60.

6. Deacetyliertes, geklärtes Heteropolysaccharid S-60, enthaltend 2% Protein, Kohlenhydrat und einen Acetylgehalt von 0%, wobei der Kohlenhydratanteil 22% Uronsäure und die neutralen Zucker Rhamnose und Glukose im ungefähren Molverhältnis 1,5:1 enthält, und erhalten aus dem im Anspruch 1 beanspruchten Heteropolysaccharid S-60.

7. Verfahren zur Herstellung der Verbindung nach Anspruch 5 durch Erwärmen einer 1—5% wässrigen Lösung von Heteropolysaccharid S-60, wie im Anspruch 1 beansprucht, bei einem pH von etwa 10 und bei einer Temperatur von 90—100°C während 10 Minuten bis 45 Minuten, und Gewinnen des dabei erzeugten Produkts.

8. Mikrobiologisches Kulturmedium enthaltend 0,5—5% des deacetylierten Produkts aus dem Heteropolysaccharid S-60, beansprucht im Anspruch 1, und einen Nährstoff.

9. Medium nach Anspruch 8, in dem das deacetylierte Heteropolysaccharid S-60 geklärt ist.

10. Duftende Gelzusammensetzung, enthaltend 0,1—5% des deacetylierten Produkts aus dem Heterpolysaccharid S-60, beansprucht im Anspruch 1, Wasser und ein niedriges Alkanol.